

Immunocamouflage of latex surfaces by grafted methoxypoly(ethylene glycol) (mPEG): Proteomic analysis of plasma protein adsorption

LE Yevgeniya^{1,2,3}, LI Li^{1,2}, WANG DunCheng^{1,2} & SCOTT Mark D.^{1,2,3*}

¹Canadian Blood Services, Vancouver V6T 1Z3, Canada;

²Centre for Blood Research, University of British Columbia, Vancouver V6T 1Z3, Canada;

³Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver V6T 1Z3, Canada

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Grafting of methoxypoly(ethylene glycol) (mPEG) to cells and biomaterials is a promising non-pharmacological immunomodulation technology. However, due to the labile nature of cells, surface-plasma interactions are poorly understood; hence, a latex bead model was studied. PEGylation of beads resulted in a density and molecular weight dependent decrease in total adsorbed protein with a net reduction from (159.9 ± 6.4) ng cm⁻² on bare latex to (18.4 ± 0.8) and (52.3 ± 5.3) ng cm⁻² on PEGylated beads (1 mmol L⁻¹ of 2 or 20 kD SCmPEG, respectively). SDS-PAGE and iTRAQ-MS analysis revealed differential compositions of the adsorbed protein layer on the PEGylated latex with a significant reduction in the compositional abundance of proteins involved in immune system activation. Thus, the biological efficacy of immunocamouflaged cells and materials is mediated by both biophysical obfuscation of antigens and reduced surface-macromolecule interactions.

PEGylation, immunocamouflage, iTRAQ, mass spectrometry, polystyrene latex, protein adsorption, proteomics, methoxypoly(ethylene glycol), polymer

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An increasing number of diverse and broad ranging clinical interventions utilize biologically derived products and/or biomaterials as therapeutic agents. Traditional biological products include blood and blood components and donor-derived tissues for transplantation while newer biologicals encompass purified proteins, cell-based therapies and, potentially, gene therapy. Biomaterials, which can be natural or synthetic, are increasingly used in medical devices that interface with biological systems such as replacement joints, stents and catheters [1,2]. However, for all of these materials, biocompatibility is a key issue, and prevention of immune recognition and subsequent host responses is crucial to avoid failure of the therapy and serious medical con-

sequences.

To achieve biocompatibility, biomaterials are often surface-modified in order to minimize protein interaction and protein adsorption to the material, thus attenuating immune recognition and rendering the surface ‘non-fouling’. A similar concept of immunomodulation can be applied to biological products. Immunocamouflage of cells and tissues refers to the biophysical and biological camouflage of cell surfaces from interaction with other cells, macromolecules and viruses [3–6]. This “biomaterials” approach for tissue engineering has emerged as a promising technique for preventing allorecognition of foreign cells, particularly in the context of transfusion and transplantation medicine. Immunocamouflage of cells is produced by the covalent grafting of methoxypoly(ethylene glycol) (mPEG; PEGylation),

*Corresponding author (email: mdscott@mail.ubc.ca)

or other low immunogenic polymers, to proteins expressed on the cell membrane. Consequent to grafting, the polymer camouflages both antigenic sites and surface charge attenuating the risk of immune recognition. This non-pharmacological technology holds great promise in preventing immunological rejection of transplanted cells and tissues without the inherent toxicity and health risks associated with currently used immunosuppressive therapies. Indeed, this tissue bioengineering approach has been experimentally shown to prevent antibody binding, reduce allorecognition and immunogenicity and to effectively block receptor-ligand interactions necessary for viral invasion while having minimal effects on cell viability and function [6].

However, due to the labile nature of biological cells, the effects of the grafted polymer on cell-plasma interactions have not been fully elucidated. To more fully examine the effect of polymer grafting on surface-macromolecule interactions between blood plasma and the surface of both biological cells and biomaterials, a latex bead (1.2 or 8.0 μm diameter) model was utilized. The effects of mPEG grafting concentration and molecular weight on total as well as specific plasma protein adsorption to the latex surface were examined via flow cytometry, quantitative protein assay, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Moreover, more detailed studies were carried out using isobaric tags for relative and absolute quantitation (iTRAQ) labeling followed by mass spectrometry (MS) to directly assess differential plasma protein adsorption to unmodified and PEGylated latex bead surfaces.

The results of these studies have provided significant new information on the interaction between human plasma and mPEG-bioengineered surfaces.

1 Materials and methods

1.1 Latex bead model and polymer grafting

To investigate the effect of PEGylation on plasma protein-surface interactions, aliphatic amine polystyrene latex beads (1.2 and 8.0 μm in diameter; Molecular Probes, Invitrogen, Carlsbad, CA, USA) were used. As shown in Figure 1, the bead consists of a hydrophobic polystyrene core and is negatively charged due to carboxyl groups (COO^-). The surface of the latex particles is functionalized with primary amine (NH_3^+) groups covalently attached to the latex core via a six-carbon aliphatic arm with an average of 3.5×10^7 residues per bead. These primary amine groups are the sites used for the covalent grafting of the activated mPEG polymer.

Activated succinimidyl carbonate methoxypoly(ethylene glycol) (SC-mPEG) (Laysan Bio, Inc., Arab, AL, USA) was dissolved in PEG buffer (50 mmol L^{-1} K_2HPO_4 , 105 mmol L^{-1} NaCl , pH 8.0) and added to a 2.0 weight percent (% w/v) aqueous suspension of aliphatic amine polystyrene latex particles to reach the desired final mPEG grafting concentration (0–5 mmol L^{-1}). Samples were incubated for 60 min at room temperature with constant mixing and subsequently

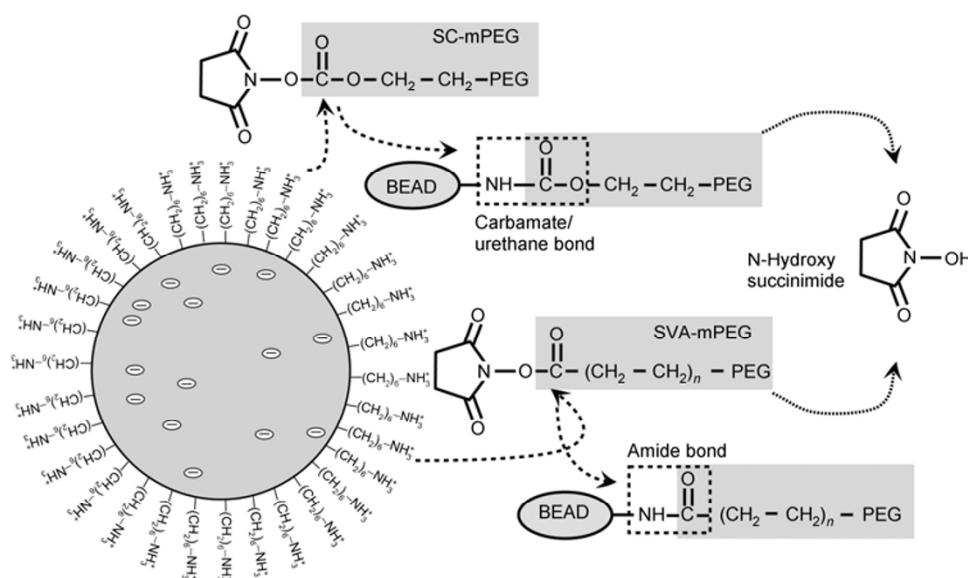


Figure 1 Latex bead model. Aliphatic amine polystyrene latex particles were utilized for plasma protein adsorption assays. The core of the particle is negatively charged and composed of hydrophobic polystyrene. The surface of the latex is functionalized with aliphatic primary amine groups. The number of positively charged amine groups is in large excess to the number of negatively charged carboxyl groups in the latex core imparting an overall positive charge to the particle. Approximately 3.5×10^7 and 7.6×10^9 amine groups are present on the 1.2 and 8.0 diameter μm particles, respectively. These primary amine groups on the latex particles (or membrane protein lysine residues) act as nucleophiles in an $\text{S}_\text{N}1$ substitution reaction, releasing the free N-hydroxy succinimide linker group and forming a covalent bond between the polymer and bead. SC-mPEG forms a carbamate (urethane) bond while SPA- and SVA-mPEG form an amide bond.

washed five times to remove unbound mPEG. As shown in Figure 1, SC-mPEG grafting resulted in a carbamate linkage between the mPEG polymer and the bead with a by-product of N-hydroxy succinimide that was subsequently removed via washing. A final aqueous latex suspension was made (2.0% w/v) and stored at 4°C for the subsequent experiments.

1.2 Plasma adsorption to and desorption from control and modified latex

Following informed consent, whole blood from healthy volunteers was collected into EDTA anticoagulant vacuum tubes. Human plasma was obtained by centrifugation at 1000×g (22°C; 5 min). Plasma protein adsorption studies were carried out using both unlabeled and fluorescently labeled plasma.

All results were expressed as mean±standard error of the mean (SEM). A minimum of three replicate experiments was performed for all studies. Statistical analyses were done using SPSS v.16.0 statistical software (Statistical Products and Services Solutions, SPSS Inc., Chicago, IL, USA). For comparison of three or more mean values, a one-way analysis of variance (ANOVA) was performed followed by a Tukey post-hoc test for pair-wise comparisons of means. Significance was determined by a two-tailed *P*-value<0.05.

1.3 Plasma protein labeling, adsorption and desorption studies

Unlabeled plasma protein adsorption experiments were performed in a 50% plasma-latex suspension (500 cm² total latex surface area). Fluorescent plasma protein adsorption studies were conducted on 83.3 cm² latex using 83.3% (diluted with unlabeled plasma) Alexa 488 (Molecular Probes, Invitrogen, Carlsbad, CA, USA) labeled plasma. Bare and differentially modified beads were incubated with plasma (unlabeled or fluorescent) for 60 min at room temperature with constant mixing. Following adsorption, samples were washed five times in 0.02 mol L⁻¹ phosphate buffer with 3 mmol L⁻¹ NaN₃ (pH 7.4) to remove unbound protein.

Latex samples with adsorbed fluorescent protein were evaluated via microscopic analysis and flow cytometry (BD FACSCalibur, BD Biosciences, San Jose, CA, USA) to qualitatively and quantitatively assess the adsorbed protein. Data acquisition and analysis were performed using CellQuest Pro software (BD Biosciences, San Jose, CA, USA) and excitation and emission wavelengths of 488 nm and 530 nm, respectively. A minimum of 20000 events was collected per sample.

Bound proteins were desorbed from bare control and differentially modified PEGylated latex beads by the addition of solubilization buffer (2% sodium dodecyl sulfate (SDS) and 80 mmol L⁻¹ dithiothreitol (DTT) in water) and incubated for 5 min in a 95°C water bath. Latex samples

were centrifuged for 5 min at 16000×g and the supernatants containing desorbed unlabeled proteins were subjected to colorimetric protein assay, SDS-PAGE, and mass spectrometry analysis. Following desorption of fluorescent protein, latex beads were evaluated via flow cytometry (as described above) to estimate the level of residual fluorescence due to “permanently” adsorbed fluorescent protein.

1.4 Quantitative protein assay

Unlabeled proteins were desorbed from 500 cm² of latex surface with 70 μL of solubilization buffer. Duplicate 25 μL samples of solubilized desorbed protein were subjected to colorimetric protein assay (RC DC protein assay, Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to manufacturer's instructions. For each assay, a standard curve was constructed using a bovine serum albumin (BSA) solution (2.0 mg mL⁻¹, Thermo Fisher Scientific, Inc., Rockford, IL, USA).

1.5 SDS-PAGE analysis

Unlabeled proteins desorbed from control and PEGylated latex beads were analyzed by SDS-PAGE as described by Laemmli [7]. Identical volumes of desorbed proteins and control solutions were loaded onto a 7% polyacrylamide gel. Electrophoresis was performed at room temperature, 25 mA current, for 60 min. Each gel included lanes containing: sample buffer only; molecular weight standards (Kaleidoscope precision plus protein standards; Bio-Rad Laboratories, Inc., Hercules, CA, USA); and 100 times diluted neat human plasma in sample buffer. Following SDS-PAGE, gel was stained with Pierce ImperialTM protein stain (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

1.6 iTRAQ and mass spectrometry

Based on the protein assay data, 200 μg of unlabeled protein was desorbed from an estimated amount of latex using 200 μL of desorption buffer. The supernatants containing desorbed protein were submitted to the University of Victoria Proteomics Centre (www.proteincentre.com) for iTRAQ labeling and MS analysis. Briefly, four protein samples desorbed from four differentially modified latex particle preparations (0 (control), 0.5, 2.0 and 5.0 mmol L⁻¹), were digested with trypsin and labeled with the iTRAQTM reagent (Applied Biosystems, Foster City, CA, USA). Differentially labeled peptides were subjected to liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) analysis using an integrated Famos autosampler, a SwitchosII switching pump and an UltiMate micro pump (LC Packings—A Dionex Company, Sunnyvale, CA, USA). The system was equipped with a Hybrid Quadrupole-Time of Flight (TOF) LC/MS/MS mass spectrometer (QStar, Mary Esther, FL, USA), a nano-electrospray ionization source

(Proxeon, Odense, Denmark) and a 10 μm fused-silica emitter tip (New Objective, Inc., Woburn, MA, USA). Mass spectrometry data were processed using Protein Pilot software (v.2.0, Applied Biosystems, Foster City, CA, USA). To identify and quantitate the relative amounts of adsorbed proteins, three stringent criteria were applied to the analysis: 99.0% confidence in peptide identification; a change in relative binding of $P \leq 0.01$ to demonstrate significance; and a minimum of three distinct peptides detected for each protein identified.

2 Results

2.1 Protein adsorption and desorption

The amount of total protein adsorbed to differentially modified latex particles (0–2 mmol L^{-1} ; 2, 5 and 20 kD SC-mPEG) was quantified by colorimetric protein assay of supernatants containing latex desorbed proteins. As shown in Figure 2A, PEGylation of latex beads resulted in an mPEG dose-dependent decrease in total plasma protein adsorption. SC-mPEG modified latex (2 mmol L^{-1} , 2 kD SC-mPEG) exhibited a dramatically decreased protein adsorption relative to the bare latex control: (15.2 ± 2.0) vs. (159.9 ± 6.4) ng cm^{-2} , respectively. Moreover, low molecular weight polymers more effectively prevented protein adsorption than high molecular weight mPEG (e.g., 2 vs. 20 kD; 0.1–2.0 mmol L^{-1} , $P \leq 0.04$). Specifically, 0.5 mmol L^{-1} 2 kD mPEG resulted in (24.3 ± 3.6) vs. (70.4 ± 9.6) ng cm^{-2} obtained with the 20 kD polymer at the same grafting concentration. Finally, only grafted mPEG was capable of providing surface camouflage and preventing protein adsorption. Latex particles incubated with soluble, non-activated mPEG showed a similar degree of protein adsorption

to that of bare latex.

The observed decreased total protein adsorption and the dose and polymer length dependency of surface camouflage were further supported by the flow cytometry studies (Figure 2B). Differentially modified latex particles were incubated with fluorescently labeled plasma and subjected to flow cytometry before and after protein desorption. The mean particle fluorescence of PEGylated latex was expressed relative to the fluorescence of unmodified latex that was normalized to 100%. These findings also demonstrated that the protein desorption process was effective at removing most but not all of the adsorbed species (Figure 2B). The values for fluorescent intensity of latex particles before vs. after protein desorption were dramatically decreased (e.g., 100.0% vs. $12.0\% \pm 2.1\%$ for bare latex and $29.9\% \pm 3.8\%$ vs. $4.7\% \pm 1.6\%$ for 0.5 mmol L^{-1} , 5 kD SC-mPEG modified beads). Surprisingly, latex fluorescence following protein desorption from either bare or modified particles remained above that of the negative control (0%), suggesting that desorption was incomplete.

To further investigate decreased protein adsorption on PEGylated surfaces, proteins desorbed from differentially modified latex particles (0–2 mmol L^{-1} , 5 kD SC-mPEG) were analyzed by SDS-PAGE (Figure 3). As demonstrated above, absolute protein adsorption decreased dramatically, and in a grafting concentration dependent manner, in the PEGylated bead samples. Consistent with those findings, incubation of latex with unactivated (i.e., linker free) soluble 5 kD mPEG (2.0 mmol L^{-1}) did not prevent protein adsorption. The interaction of plasma proteins with control or PEGylated beads was further demonstrated microscopically using control and PEGylated 8.0 μm latex beads incubated with Alexa Fluor 488-labeled human plasma. As shown in Figure 4, control beads avidly bound human plasma while the polymer grafted beads demonstrated a very significant

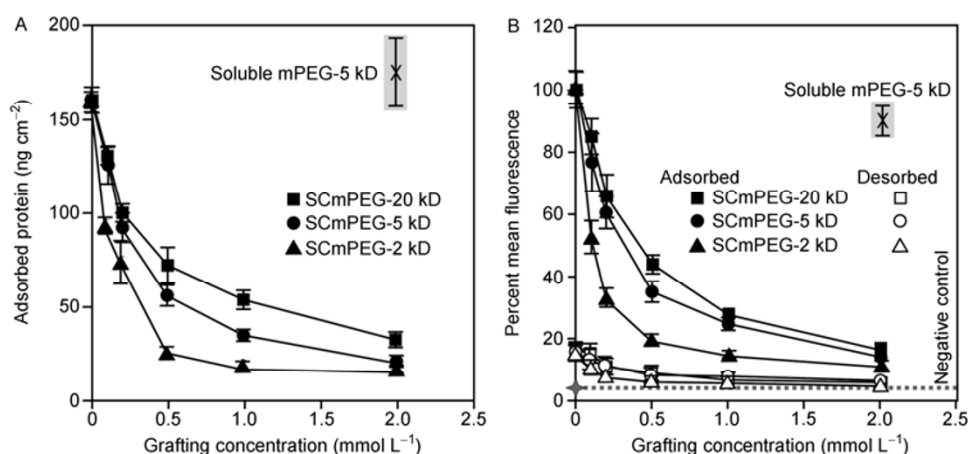


Figure 2 PEGylation of 1.2 μm latex with SC-mPEG resulted in the dramatic reduction of the total amount of plasma protein adsorbed to the modified beads. Plasma protein-surface interactions were significantly inhibited by grafted mPEG in a dose-dependent manner as measured quantitatively by colorimetric protein assay (A) and flow cytometry (B). Shorter polymers (2 kD) were shown to have increased efficacy of biophysical surface camouflage in comparison to the longer polymers (20 kD), especially at lower grafting concentrations. The protein desorption protocol resulted in a substantial but incomplete removal of all adsorbed species.

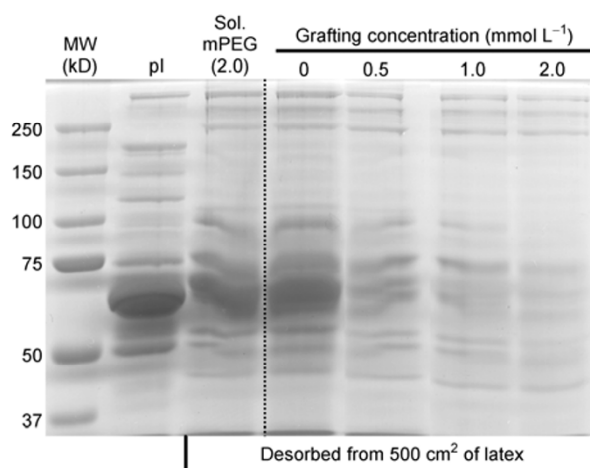


Figure 3 PEGylation of 1.2 μm beads not only decreased total protein adsorption, but also resulted in a differential composition of adsorbed protein layer. SDS-PAGE analysis of proteins desorbed from differentially modified latex (0, 0.5, 2, 5 mmol L^{-1} 5 kD SC-mPEG) demonstrated a non-uniform decrease in protein band intensity. MW, Kaleidoscope multi-color molecular weight markers; PI, 100 times diluted human plasma; Sol. 2.0, protein taken off latex incubated with soluble 2.0 mmol L^{-1} , 5 kD mPEG incapable of covalent binding. Protein was desorbed as described in Methods. The SDS-PAGE reflects a lower resolution analysis of protein adsorption versus that provided by iTRAQ analysis (Tables 1–4).

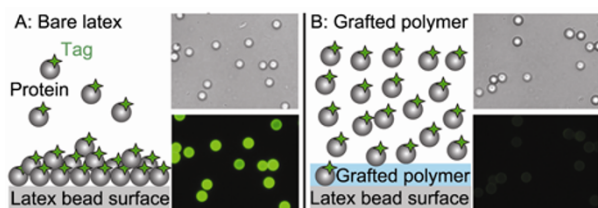


Figure 4 Adsorption of Alexa Fluor 488-labeled human plasma to 8.0 μm latex beads was significantly ($P < 0.001$) reduced by grafted polymer. Shown schematically and microscopically is the interaction of fluorescently tagged plasma proteins with the surface of control (A) or PEGylated (B) 8.0 μm latex beads. Photomicrographs: light microscopy image (upper panel); fluorescent microscopy image (lower panel).

($P < 0.0001$) decrease in protein adsorption.

2.2 iTRAQ analysis

As shown in Figures 2 and 3, total protein adsorption to the PEGylated beads was dramatically reduced. However, as can be observed in Figure 3, not all proteins demonstrated a proportional decrease in adsorption to the PEGylated samples relative to the total decrease in adsorption. This was indicated by the variability and a non-uniform decrease in intensity of specific protein bands. This, visual observation indicated a differential protein composition of the adsorbed layer on PEGylated vs. bare latex surfaces. Thus, the composition and the relative abundance of specific proteins within the total adsorbed protein layer were studied by subjecting desorbed protein samples to iTRAQ labeling and

MS analysis [8]. For analytic analysis, the iTRAQ/MS protocol required that all the protein samples subjected to the labeling contain the same amount of total protein (100 μg) in order to normalize the samples and avoid discrepancy during the MS analysis. Since the total protein adsorption was dramatically decreased on the PEGylated latex surface, iTRAQ analysis required 3, 9 and 15 times more of the 0.5, 2.0 and 5.0 mmol L^{-1} modified latex beads (1.2 μm ; 5 kD SC-mPEG) than the unmodified latex control in order to obtain the required 100 μg of desorbed protein.

The comparative MS analysis identified 95 protein species adsorbed to the unmodified latex samples (Table 1). Because human plasma preparations contained low amounts of proteins derived from tissues and residual white cells, platelets and erythrocytes, a plasma protein subset consisting of 47 proteins was identified. A second subset of 32 immune regulatory plasma proteins was further delineated from this population based on well-characterized protein functions and consisted primarily of complement and coagulation proteins as well as a small number of other proteins. The relative abundance of the desorbed protein within the total and plasma protein subsets differentially varied between bare and PEGylated beads. For the PEGylated (relative to bare) beads, 32 of the 47 plasma proteins demonstrated decreased adsorption, five proteins exhibited a relative (to total) increase in abundance, while 10 remained unchanged when compared to the adsorbed protein composition of the bare latex control (Table 1). Moreover, of the immune regulatory plasma protein subset (32 proteins total), the majority exhibited a significant reduction in relative adsorption (Table 1; Figure 5).

As demonstrated in Tables 2A–C, 68% of the plasma proteins exhibited significantly reduced relative adsorption to the PEGylated beads. Moreover, 62% of the immune regulatory proteins also exhibited decreased abundance (Figure 5; Table 2). Importantly, these proteins participate in the primary events related to the discrimination between “self” and “non-self” as well as activation of the immune system. Decreased relative adsorption of these pro-activating proteins on foreign substrates is of primary importance and renders such surfaces “non-fouling” or “stealth”. For example, prothrombin, fibrinogen, factor V and plasminogen activator inhibitor are important mediators of the coagula-

Table 1 Summary of iTRAQ/MS Analysis. Shown are the numbers and the types of proteins demonstrating decreased, increased or unchanged abundances in the protein layer adsorbed to PEGylated beads (relative to unmodified latex beads)

	Total	Plasma proteins	Immune proteins subset
Identified	95	47	32
Decreased abundance on PEGylated surface	43	32	20
Increased abundance on PEGylated surface	15	5	6
Unchanged abundance on PEGylated surface	37	10	6

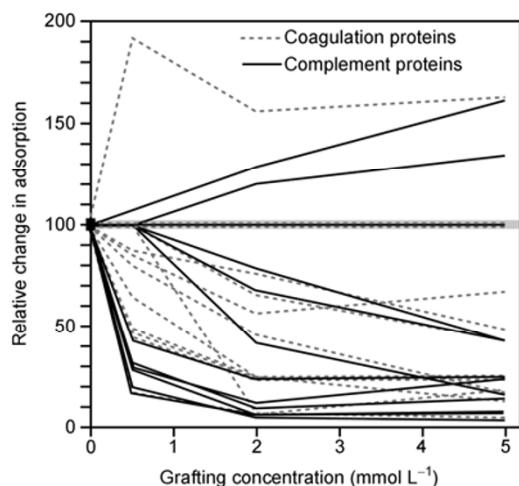


Figure 5 Complement and coagulation related proteins exhibited a predominant trend of significantly reduced adsorption. Data shown represents the percent change in adsorption (relative to unmodified latex) of the proteins listed in Tables 2–4.

tion pathway. C4b, LPS binding protein, C1s, C1r, C3, C9 and MASP-3 are potent activators of complement. Decreased abundance on PEGylated surfaces was also observed for proteins that serve inhibitory functions in the immune activation (e.g., plasminogen, C4b binding protein, vitamin K-dependent protein S and heparin cofactor II).

In contrast, increased abundance on modified latex was noted for 5 (i.e., 10.6%) plasma proteins (Table 3). Four of these species are involved in immune regulation: C1q (alpha and beta chains), a fragment of a constant chain of IgG immunoglobulin, thrombospondin 1 and beta-2-glycoprotein. Finally, only 10 (i.e., 21.3%) plasma proteins (Table 4) demonstrated equivalent adsorption to both the PEGylated and unmodified beads. Most of these species serve homeostatic functions, such as binding and transport, and include serum albumin, multimerin 1 and apolipoproteins A-V, C-II, C-III. Not unexpectedly, albumin constituted the vast majority of adsorbed protein in this group. Only four of the 10 proteins are involved in immune regulation: C1q (C-chain),

Table 2 Decreased relative plasma protein adsorption of coagulation-related, complement-related, and other proteins. Values presented are percent decrease in abundance on PEGylated latex beads (relative to unmodified beads). Proteins were identified and quantified by iTRAQ/MS analysis^{a)}

Accession	MW (kD)	pI	Protein name	0.5 mmol L ⁻¹	2 mmol L ⁻¹	5 mmol L ⁻¹
Coagulation-related proteins						
IPI:IPI00019568.1	65.3	5.2	Prothrombin precursor	20.2 ^{##}	55.4 ^{**}	81.9 [*]
IPI:IPI00021885.1	75.5	5.1	Fibrinogen alpha/alpha-E chain precursor	56.4 ^{**}	77.4 [*]	77.7 [*]
IPI:IPI00298497.3	50.8	8.0	Fibrinogen beta chain precursor	51.3 ^{**}	74.9 [*]	75.3 [*]
IPI:IPI00019580.1	88.4	7.1	Plasminogen precursor	34.7 [#]	74.4 ^{**}	87.0 [*]
IPI:IPI00219713.1	48.5	5.2	Fibrinogen gamma chain precursor	53.7 ^{**}	75.5 [*]	75.5 [*]
IPI:IPI00294004.1	70.6	5.2	Vitamin K-dependent protein S precursor	0.0 ^{##}	93.6 [*]	95.5 [*]
IPI:IPI00478809.3	248.7	5.7	Coagulation factor V precursor	14.0 ^{##}	42.8 [#]	33.4 [#]
IPI:IPI00292950.3	55.0	6.3	Heparin cofactor II precursor	82.2 [*]	94.6 [*]	82.3 [*]
IPI:IPI00007118.1	42.8	7.0	Plasminogen activator inhibitor-1 precursor	13.5 ^{##}	23.1 [#]	51.5 ^{**}
IPI:IPI00216065.1	40.4	5.3	Vitamin K-dependent protein Z precursor	0.0 ^{##}	36.0 [#]	58.0 ^{**}
Complement-related proteins						
IPI:IPI00453459.1	192.8	6.9	Complement Component 4B preproprotein	83.4 [*]	94.6 [*]	94.5 [*]
IPI:IPI00021727.1	61.7	6.2	C4b-binding protein alpha chain precursor	71.4 ^{**}	93.3 [*]	93.6 [*]
IPI:IPI00400826.1	50.1	5.9	Clusterin	0.0 ^{##}	21.2 [#]	58.6 ^{**}
IPI:IPI00025862.1	26.4	5.0	C4b-binding protein beta chain precursor	80.4 [*]	96.7 [*]	97.6 [*]
IPI:IPI00017696.1	74.9	4.9	Complement C1s subcomponent precursor	0.0 ^{##}	31.3 [#]	57.9 ^{**}
IPI:IPI00164623.3	185.0	6.0	Complement C3 precursor	58.1 ^{**}	75.5 [*]	75.9 [*]
IPI:IPI00022395.1	61.0	5.4	Complement component C9 precursor	71.0 ^{**}	89.4 [*]	78.5 [*]
IPI:IPI00290283.5	81.9	5.0	Complement factor MASP-3	0.0 ^{##}	59.5 ^{**}	86.7 [*]
IPI:IPI00296165.5	78.3	5.8	Complement C1r subcomponent precursor	69.9 ^{**}	90.7 [*]	86.8 [*]
Other proteins						
IPI:IPI00022229.1	512.8	6.6	Apolipoprotein B-100 precursor	31.3 [#]	82.2 [*]	53.7 ^{**}
IPI:IPI00021841.1	28.1	5.3	Apolipoprotein A-I precursor	19.4 ^{##}	51.2 ^{**}	63.6 ^{**}
IPI:IPI00304273.1	43.4	5.2	Apolipoprotein A-IV precursor	29.8	51.2 ^{**}	34.4 [#]
IPI:IPI00218192.1	101.2	6.2	Inter-alpha-trypsin inhibitor H4 precursor	50.1 ^{**}	64.1 ^{**}	57.1 ^{**}
IPI:IPI00514475.3	41.1	5.7	OTTHUMP00000028705	41.5 [#]	62.3 ^{**}	70.9 ^{**}
IPI:IPI00021842.1	34.2	5.5	Apolipoprotein E precursor	23.7 [#]	43.6 [#]	57.3 ^{**}
IPI:IPI00028413.3	69.4	5.0	Inter-alpha inhibitor H3	0.0 ^{##}	81.9 [*]	78.1 [*]
IPI:IPI00292530.1	71.4	6.3	Inter-alpha-trypsin inhibitor H1 precursor	78.2 [*]	94.2 [*]	84.2 [*]
IPI:IPI00305461.2	72.4	5.8	Inter-alpha-trypsin inhibitor H2 precursor	73.4 ^{**}	88.1 [*]	79.6 [*]
IPI:IPI00554598.1	23.8	4.8	Hypothetical protein DKFZp686N1868	0.0 ^{##}	42.1 [#]	77.9 [*]
IPI:IPI00298971.1	52.3	5.5	Vitronectin precursor	3.6 ^{##}	43.4 [#]	71.3 ^{**}
IPI:IPI00218732.2	39.6	5.1	Serum paraoxonase/arylesterase 1	0.0 ^{##}	24.8 [#]	54.1 ^{**}
IPI:IPI00032311.3	50.9	6.3	Lipopolysaccharide-binding protein precursor	70.9 ^{**}	85.1 [*]	54.3 ^{**}

a) Values are expressed as a percent decrease in protein abundance per 100 µg of total protein desorbed from PEGylated beads (relative to bare latex control). Percent decreased abundance labels: *, >75%; **, 50%–74.9%; #, 20%–49.9%; ##, <20%. Bold denotes immune-relevant proteins. IPI, international protein index; MW, molecular weight; kD, kilodaltons; pI, isoelectric point.

Table 3 Increase in relative plasma protein adsorption. Values presented are percent increase in abundance on PEGylated latex beads (relative to unmodified beads). Proteins were identified and quantitated by iTRAQ/MS analysis^{a)}

Accession	MW (kD)	pI	Protein name	Percent increased adsorption			Class
				0.5 mmol L ⁻¹	2 mmol L ⁻¹	5 mmol L ⁻¹	
IPI:IPI00296099.3	127.5	4.7	Thrombospondin-1 precursor	5.2 ^{##}	18.3 ^{##}	26.3 [#]	E.C. matrix
IPI:IPI00549769.1	36.1	8.5	Ig gamma-1 chain C region	20.0 ^{##}	0.0 ^{##}	89.9 [*]	Immune
IPI:IPI00022392.1	23.7	9.3	Complement C1q, A chain precursor	0.0 ^{##}	20.2 ^{##}	34.4 [#]	Complement
IPI:IPI00477992.1	23.7	8.9	Complement component 1, q subcomponent, beta polypeptide	0.0 ^{##}	28.1 [#]	60.4 ^{**}	Complement
IPI:IPI00298828.1	36.3	8.4	Beta-2-glycoprotein I precursor	94.9 [*]	55.2 ^{**}	64.7 ^{**}	Coagulation

a) Values are expressed as a percent increase in protein abundance per 100 μ g of total protein desorbed from PEGylated beads (relative to bare latex control). Percent decreased abundance labels: *, >75%; **, 50%–74.9%; #, 20%–49.9%; ##, <20%. Bold denotes immune-relevant proteins. IPI, international protein index; MW, molecular weight; kD, kilodaltons; pI, isoelectric point.

Table 4 Unchanged relative plasma protein adsorption. Plasma proteins exhibiting unchanged abundance on PEGylated latex (relative to unmodified beads). Proteins were identified and quantitated by iTRAQ/MS analysis^{a)}

Accession	MW (kD)	pI	Protein name	Class
IPI:IPI00022434.1	66.5	5.7	Serum albumin precursor	Transport
IPI:IPI00303283.1	84.4	5.0	Integrin beta-3 precursor	Receptor
IPI:IPI00418392.1	136.1	7.9	Multimerin 1	Transport
IPI:IPI00022394.2	22.8	8.3	Complement C1q subcomponent, C chain precursor	Complement
IPI:IPI00023014.1	225.7	5.4	Von Willebrand factor precursor	Coagulation
IPI:IPI00465378.1	38.9	6.0	Apolipoprotein A-V precursor	Transport
IPI:IPI00021857.1	8.8	4.7	Apolipoprotein C-III precursor	Transport
IPI:IPI00022445.1	10.3	9.0	Platelet basic protein precursor	Immune
IPI:IPI00022446.1	7.8	8.8	Platelet factor 4 precursor	Coagulation
IPI:IPI00021856.3	8.9	4.7	Apolipoprotein C-II precursor	Transport

a) Although there was no difference in the relative abundance of the above-listed species per 100 μ g total desorbed protein solution from either bare or modified latex, the total adsorption to PEGylated surfaces decreased dramatically from that of unmodified beads, e.g., (18.3 \pm 4.1) (2 mmol L⁻¹, 5 kD SC-mPEG) vs. (159.9 \pm 6.4) ng cm⁻² (bare latex). Bold denotes immune-relevant proteins. IPI, international protein index; MW, molecular weight; kD, kilodaltons; pI, isoelectric point.

Von Willebrand factor, platelet basic protein and platelet factor 4.

3 Discussion

Immunologic recognition of foreign materials and cells is highly dependent on cell surface interactions with either soluble factors (e.g., antibody binding or denaturation of plasma proteins) and/or recognition by cellular components of the immune system (e.g., antigen presenting cells and lymphocytes). Previous studies have demonstrated that easily denatured (i.e., “soft”) proteins are capable of unfolding and binding to hydrophobic surfaces via multiple contacts upon exposure of internal hydrophobic residues. This leads to very strong adhesion through additional H-bonds, hydrophobic, charge-charge and Van der Waals interactions [9,10]. The use of grafted polymers to immunocamouflage the surface of implanted biomaterials, transplanted tissues or transfused cells targets this initial, and crucial, step in the process of inflammation and allorecognition in a manner that is applicable to a broad range of clinical scenarios.

In this study, a latex bead model was utilized to examine the binding of plasma proteins to PEGylated surfaces. The

covalent grafting of mPEG to latex particles resulted in a significant decrease in surface-macromolecule interactions (Figures 2–5) resulting in the global immunocamouflage of the particles, as indicated by the decreased total plasma protein adsorption. However, in contrast to previous biological studies, the biophysical latex model together with the application of MS-based proteomics allowed for the quantitative and qualitative estimations of total as well as specific surface interactions inhibited by PEGylation, aiding in the further elucidation of biological responses to PEGylated surfaces.

mPEG is a highly flexible, hydrophilic polymer occupying a large 3D space (i.e., possessing a large exclusion volume) and can dramatically alter the characteristics of any surface it is bound to (Figure 6A). Chemically, due to the highly hydrophilic nature of mPEG and its extensive water binding properties, PEGylation results in increased surface hydrophilicity of the latex particles [11]. Previous studies have shown that hydrophilic surfaces have decreased total protein adsorption and are also more biocompatible [12]. Thus, mPEG grafting generates a steric barrier while simultaneously biophysically camouflaging surface charge thus reducing immune recognition of implantable biomaterials, allogeneic red blood cells [5,13–18], lymphocytes [3,4,19–21]

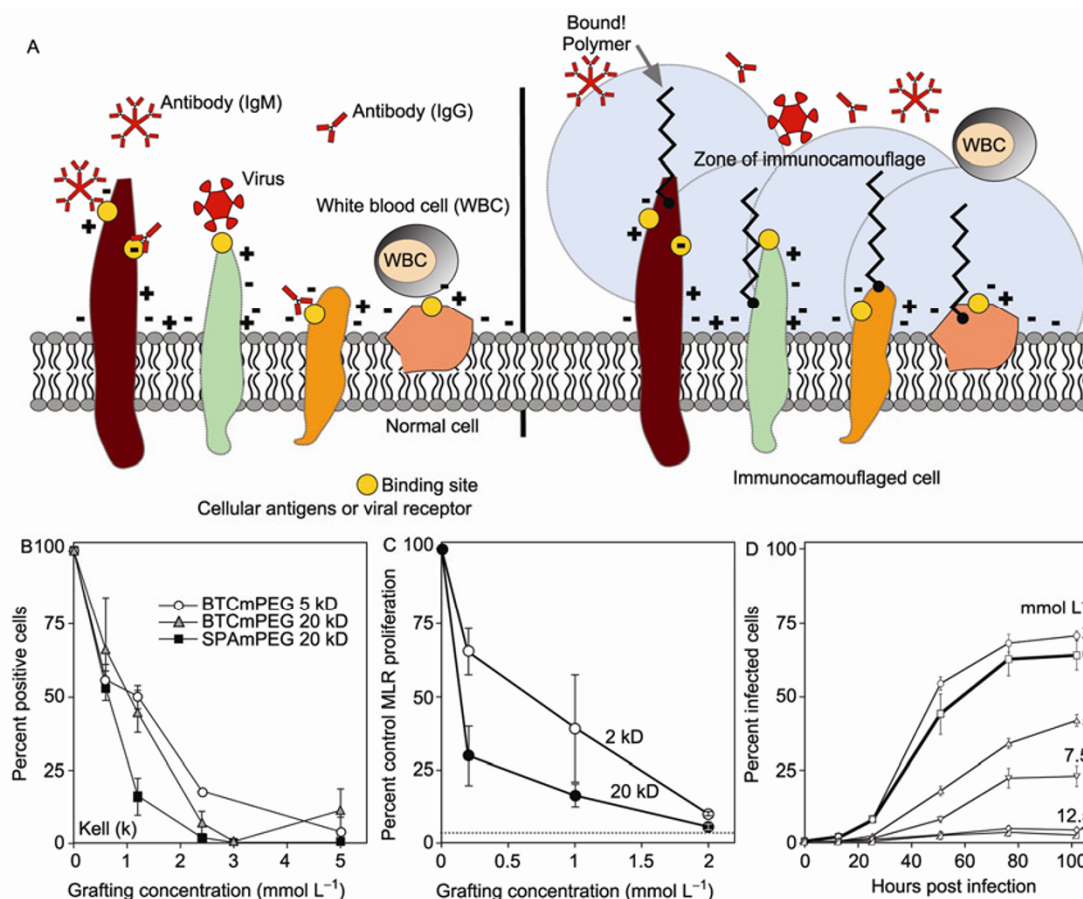


Figure 6 The reduced interaction of plasma proteins with latex beads is highly predictive of the biological utility of immunocamouflage. Not only can grafted polymers prevent unwanted adsorption of plasma proteins to biomaterials (e.g., vascular stents), they can also prevent or attenuate undesirable biological interactions. A, The covalent grafting of polymers (e.g., mPEG or HPG) resulted in the biophysical camouflage of surface proteins (e.g., cell receptors, antigenic site and viral binding sites). This was accomplished via both a steric barrier and charge camouflage. B, Consequent to this biophysical barrier, RBC exhibited reduced antibody binding to blood group antigens (e.g., Kell, k). C, Immunocamouflage of lymphocytes resulted in the loss of allorecognition of MHC disparate cells as demonstrated by attenuated proliferation in mixed lymphocyte reactions (MLR). D, The immunocamouflage of viral receptors located on the membrane surface of host cells reduced viral invasion, proliferation and disease progression.

and viruses (Figure 6B–D) [22]. As shown in this study, consequent to PEGylation, latex particles demonstrated significant inhibition of protein adsorption in a polymer dose- and length-dependent manner, as measured quantitatively with colorimetric protein assay and flow cytometry (Figure 2A and B) and qualitatively by SDS-PAGE, fluorescent microscopy and iTRAQ-MS analysis (Figures 3–5).

The inhibition of plasma protein adsorption to latex beads was effected by changes in thermodynamics of the latex-plasma interface mediated by charge camouflage and, most notably, the steric exclusion effect of the grafted mPEG polymers. Steric effects were increased when polymers were grafted at higher density, particularly by short chain polymers (e.g., 2 kD) that can be grafted at a higher density (Figure 2A and B). In contrast to the lower molecular weight polymers, high density grafting of long chain polymers is inhibited via steric self-exclusion effects arising from the initial population of grafted polymers.

Biophysically, the steric hindrance arose due to decreased conformational freedom of the grafted mPEG polymer resulting in decreased entropy of the polymers. When macromolecules interact with mPEG-modified surfaces, they contact and compress the polymer chains resulting in a further decrease in entropy. This highly thermodynamically unfavorable state is then reduced by the rejection of molecules from the polymer contact points thereby preventing protein adsorption and increasing entropy and conformational freedom of the polymer chains [23–25]. At higher surface polymer densities, (Figures 2 and 3) chains are even more restricted in conformational freedom thereby increasing plasma protein rejection from the biomaterial (bead) surface.

As evidenced by the iTRAQ/MS studies (Tables 1–4), 68% of all identified plasma proteins exhibited reduced binding to PEGylated surfaces. Of those proteins showing reduced adsorption, the amount of bound protein reduced

was dependent on mPEG grafting concentration. For example, as shown in Table 2, 0.5, 2 and 5 mmol L⁻¹ grafting concentrations resulted in 12.5%, 50% and 56% of the identified proteins showing a $\geq 75\%$ decreased abundance in comparison to bare latex control. As demonstrated in Table 2, decreased adsorption of multiple immune proteins involved in allorecognition, coagulation and complement activation (e.g., plasminogen, C4b binding protein, vitronectin, vitamin K-dependent protein S, and heparin cofactor II) was noted. The observed reduction of adsorbed protein coupled with the increased hydrophilicity of PEGylated surfaces is also potentially beneficial as these surfaces are less thrombogenic than surfaces with adsorbed, partially denatured proteins and/or hydrophobic surfaces. Indeed, the propensity of hydrophobic surfaces to be thrombogenic is partially derived from the increased avidity for, adsorption of, and denaturation of plasma proteins.

A sparingly small number of proteins (Tables 1 and 3) exhibited a relative increase in adsorption or interaction with the PEGylated bead surface. Interestingly, these proteins were collagen or fibrin-like proteins, or proteins involved in adhesion and binding of collagen-like proteins. However, while small in number, these proteins are active components of the immune system and did demonstrate an mPEG-dose dependent increase in adsorption. Included among these proteins were components of the C1q complex (alpha and beta chains) that is capable of initiating complement activation. The C1q (alpha and beta chains) protein interacts with the constant chain of IgG [26,27] and both could become bound to the PEGylated surfaces due to C1q's collagen-like structure [28]. However, previous studies have demonstrated no direct complement activation or consumption by mPEG-modified human erythrocytes [15]. The increased relative abundance of thrombospondin 1 likely arose due to its complex multi-domain structure that allows it to interact with cell-adhesive receptors and glycoproteins, including fibronectin and collagen [29]. Finally, not all adsorbed protein would be detrimental. The binding of beta-2-glycoprotein I precursor and thrombospondin could potentially serve beneficial functions in cell and tissue transplantation. Beta-2-glycoprotein binds to negatively charged phospholipids and inhibits the coagulation cascade [30,31], while thrombospondin-1 is a potent inhibitor of T-cell and dendritic cell activation [32,33].

Of the 10 proteins (Table 4) that exhibited no relative change in abundance, the majority serve homeostatic functions (e.g., binding and transport). Only four proteins from this subset have immunological roles: C1q (c-chain), Von Willebrand factor, platelet basic protein and platelet factor 4. The c-chain of C1q is responsible for binding of the C1q complex while Von Willebrand factor acts as a chaperone for coagulation factor VIII; thus in addition to their roles as immune-related proteins, both fall into the subset of binding and transport proteins. Platelet factor 4 and platelet basic protein belong to the family of chemokines responsible for

down-regulation of immunological response. Platelet factor 4 inhibits lymphocyte proliferation and cytokine release [34]. Platelet basic protein was shown to decrease chemotaxis and function of neutrophils in pro-inflammatory response [35]. In addition, as chemokines are signaling proteins, in the absence of binding to their receptors these proteins would, most likely, not exert any biological effects. Thus, none of these proteins are implicated in the activation of the immune system and their adsorption should not result in adverse immune reactions.

Our proteomic findings are both predictive of, and supported by, biological observations. As demonstrated in Figure 6, polymer grafting to intact cells prevented or attenuated immunologically undesirable biological interactions. As schematically presented in Figure 6A, the covalent grafting of polymers (e.g., mPEG or HPG) results in the biophysical camouflage of surface proteins (e.g., cell receptors, antigenic sites and viral binding sites). Consequent to the formation of this biophysical barrier: human erythrocytes exhibit reduced antibody binding to blood group antigens (e.g., Kell (k)) (Figure 6B); allorecognition of HLA disparate lymphocytes is attenuated as demonstrated by loss of proliferation in mixed lymphocyte reactions (MLR) (Figure 6C); and viral receptors located on the membrane surface of host cells are immunocamouflaged resulting in reduced viral invasion, proliferation and disease progression (Figure 6D). In aggregate, the biophysical, proteomic and biological studies all support the potential utility of immunocamouflage in biomaterials and modified cells/tissue in clinical medicine.

In summary, covalent grafting of mPEG to latex beads gave rise to the global immunocamouflage of the bead surface resulting in decreased plasma protein adsorption as assessed by both qualitative and quantitative protein analyses (Figure 7). The reduction in plasma adsorption arises consequent to thermodynamic steric exclusion and charge camouflage effects of the grafted polymer. Moreover, quantitative proteomic studies demonstrated that latex bead modification significantly prevented surface interactions with plasma proteins involved in immune recognition of biomaterials and allogeneic cells and tissues. Biologically, the reduced, adverse, surface-macromolecule interactions theoretically should enhance biocompatibility by preventing the generation of immune recognition signals (e.g., denatured proteins or immunoglobulin binding). Indeed, these latex bead biocompatibility studies are supportive and predictive of our previously reported biologic observations (Figure 6). The knowledge derived from these proteomic studies will help further define the physical and chemical parameters for PEGylation (or other polymers) of biomaterials and allogeneic cells and tissues in order to precisely control the extent and specificity of fluid-surface (e.g., plasma-implantable material), cell-cell and cell-macromolecule interactions. The practical application of the immunocamouflage technology could have significant impact

on improving the safety of implantable biomaterials as well as benefit transfusion and transplantation medicine.

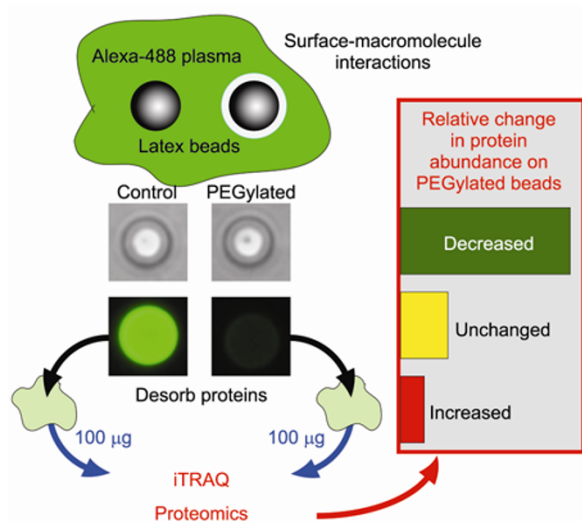


Figure 7 Qualitative and quantitative analysis of plasma surface interactions demonstrated the efficacy of immunocamouflage in preventing protein adsorption. Moreover, iTRAQ analysis provided clear evidence for reduced adsorption of proteins associated with immunological recognition of foreign cells/tissues and biomaterials.

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